DIAGNOSIS AND TREATMENT OF MALIGNANT NEOPLASMS

Statement as to Federally Sponsored Research

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Background of the Invention

10   Primary malignant central nervous system (CNS) neoplasms, particularly glioblastomas, are highly fatal due to their aggressive and widespread infiltration of the brain and resistance to anti-cancer treatments. Although progress has been made in unraveling the pathological mechanisms

15   underlying CNS cancers as well as other cancer types, tumor specific therapeutic approaches and methods of diagnosis have been largely elusive.

Summary of the Invention

The invention features a method for diagnosing a

20   malignant neoplasm in a mammal by contacting a bodily fluid from the mammal with an antibody which binds to an human aspartyl (asparaginyl) beta-hydroxylase (HAAH) polypeptide under conditions sufficient to form an antigen-antibody complex and detecting the antigen-antibody complex.

25   Malignant neoplasms detected in this manner include those derived from endodermal tissue, e.g., colon cancer, breast cancer, pancreatic cancer, liver cancer, and cancer of the bile ducts. Neoplasms of the central nervous system (CNS) such as primary malignant CNS neoplasms of both neuronal and

30   glial cell origin and metastatic CNS neoplasms are also detected. Patient derived tissue samples, e.g., biopsies of solid tumors, as well as bodily fluids such as a CNS-derived bodily fluid, blood, serum, urine, saliva, sputum, lung effusion, and ascites fluid, are contacted with an HAAH-

35   specific antibody.

The assay format is also useful to generate temporal data used for prognosis of malignant disease. A method for prognosis of a malignant neoplasm of a mammal is carried out by (a) contacting a bodily fluid from the mammal with an

5   antibody which binds to an HAAH polypeptide under conditions sufficient to form an antigen-antibody complex and detecting the antigen-antibody complex; (b) quantitating the amount of complex to determine the level of HAAH in the fluid; and (c) comparing the level of HAAH in the fluid with a normal

10   control level of HAAH. An increasing level of HAAH over time indicates a progressive worsening of the disease, and therefore, an adverse prognosis.

The invention also includes an antibody which binds to HAAH. The antibody preferably binds to a site in the

15   carboxyterminal catalytic domain of HAAH. Alternatively, the antibody binds to an epitope that is exposed on the surface of the cell. The antibody is a polyclonal antisera or monoclonal antibody. The invention encompasses not only an intact monoclonal antibody, but also an immunologically-

20   active antibody fragment, e. g. , a Fab or (Fab)$_2$ fragment; an engineered single chain Fv molecule; or a chimeric molecule, e.g., an antibody which contains the binding specificity of one antibody, e.g., of murine origin, and the remaining portions of another antibody, e.g., of human

25   origin. Preferably the antibody is a monoclonal antibody such as FB50, 5C7, 5E9, 19B, 48A, 74A, 78A, 86A, HA238A, HA221, HA 239, HA241, HA329, or HA355. Antibodies which bind to the same epitopes as those monoclonal antibodies are also within the invention.

30   An HAAH-specific intrabody is a recombinant single chain HAAH-specific antibody that is expressed inside a target cell, e.g., tumor cell. Such an intrabody binds to endogenous intracellular HAAH and inhibits HAAH enzymatic

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activity or prevents HAAH from binding to an intracellular
ligand. HAAH-specific intrabodies inhibit intracellular
signal transduction, and as a result, inhibit growth of
tumors which overexpress HAAH.

5   A kit for diagnosis of a tumor in a mammal contains
an HAAH-specific antibody. The diagnostic assay kit is
preferentially formulated in a standard two-antibody binding
format in which one HAAH-specific antibody captures HAAH in
a patient sample and another HAAH-specific antibody is used

10   to detect captured HAAH. For example, the capture antibody
is immobilized on a solid phase, e.g., an assay plate, an
assay well, a nitrocellulose membrane, a bead, a dipstick,
or a component of an elution column. The second antibody,
i.e., the detection antibody, is typically tagged with a

15   detectable label such as a colorimetric agent or
radioisotope.

          Also within the invention is a method of inhibiting
tumor growth in a mammal, which is carried out by
administering to the mammal a compound which inhibits

20   expression or enzymatic activity of HAAH. Preferably, the
compound is substantially pure nucleic acid molecule such as
an HAAH antisense DNA, the sequence of which is
complementary to a coding sequence of HAAH. Expression of
HAAH is inhibited by contacting mammalian cells, e.g., tumor

25   cells, with HAAH antisense DNA or RNA, e.g., a synthetic
HAAH antisense oligonucleotide. For example, HAAH antisense
nucleic acid is introduced into glioblastoma cells or other
tumor cells which overexpress HAAH. Binding of the
antisense nucleic acid to an HAAH transcript in the target

30   cell results in a reduction in HAAH production by the cell.
By the term "antisense nucleic acid" is meant a nucleic acid
(RNA or DNA) which is complementary to a portion of an mRNA,
and which hybridizes to and prevents translation of the

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mRNA. Preferably, the antisense DNA is complementary to the 5' regulatory sequence or the 5' portion of the coding sequence of HAAH mRNA (e.g., a sequence encoding a signal peptide or a sequence within exon 1 of the HAAH gene).

5   Standard techniques of introducing antisense DNA into the cell may be used, including those in which antisense DNA is a template from which an antisense RNA is transcribed. The method is to treat tumors in which expression of HAAH is upregulated, e.g., as a result of malignant transformation

10   of the cells. The length of the oligonucleotide is at least 10 nucleotides and may be as long as the naturally-occurring HAAH transcript. Preferably, the length is between 10 and 50 nucleotides, inclusive. More preferably, the length is between 10 and 20 nucleotides, inclusive.

15   By "substantially pure DNA or RNA" is meant that the nucleic acid is free of the genes which, in the naturally-occurring genome of the organism from which the DNA of the invention is derived, flank a HAAH gene. The term therefore includes, for example, a recombinant nucleic acid which is

20   incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a procaryote or eucaryote at a site other than its natural site; or which exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease

25   digestion) independent of other sequences. It also includes a recombinant nucleic acid which is part of a hybrid gene encoding additional polypeptide sequence such as a nucleic acid encoding an chimeric polypeptide, e.g., one encoding an antibody fragment linked to a cytotoxic polypeptide.

30   Alternatively, HAAH expression is inhibited by administering a ribozyme or a compound which inhibits binding of Fos or Jun to an HAAH promoter sequence.

Compounds, which inhibit an enzymatic activity of
HAAH, are useful to inhibit tumor growth in a mammal. By
enzymatic activity of HAAH is meant hydroxylation of an
epidermal growth factor (EGF)-like domain of a polypeptide.
For example an EGF-like domain has the consensus sequence
$CX_7CX_4CX_{10}CXCX_8C$ (SEQ ID NO:1). HAAH hydroxylase activity is
inhibited intracellularly. For example, a dominant negative
mutant of HAAH (or a nucleic acid encoding such a mutant) is
administered. The dominant negative HAAH mutant contains a
mutation which changes a ferrous iron binding site from
histidine of a naturally-occurring HAAH sequence to a non-
iron-binding amino acid, thereby abolishing the hydroxylase
activity of HAAH. The histidine to be mutated, e.g.,
deleted or substituted, is located in the carboxyterminal
catalytic domain of HAAH. For example, the mutation is
located between amino acids 650-700 (such as the His motif,
underlined sequence of SEQ ID NO:2) the native HAAH
sequence. For example, the mutation is at residues 671,
675, 679, or 690 of SEQ ID NO:2. An HAAH-specific intrabody
is also useful to bind to HAAH and inhibit intracellular
HAAH enzymatic activity, e.g., by binding to an epitope in
the catalytic domain of HAAH. Other compounds such as L-
mimosine or hydroxypyridone are administered directly into a
tumor site or systemically to inhibit HAAH hydroxylase
activity.

Table 1: Amino acid sequence of HAAH

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MAQRKNAKSS  GNSSSSGSGS  GSTSAGSSSP  GARRETKHGG  HKNGRKGGLS  GTSFFTWFMV  61
IALLGVWTSV  AVVWFDLVDY  EEVLGKLGIY  DADGDGDFDV  DDAKVLLGLK  ERSTSEPAVP  121
PEEAEPHTEP  EEQVPVEAEP  QNIEDEAKEQ  IQSLLHEMVH  AEHVEGEDLQ  QEDGPTGEPQ  181
QEDDEFLMAT  DVDDRFETLE  PEVSHEETEH  SYHVEETVSQ  DCNQDMEEMM  SEQENPDSSE  241
PVVEDERLHH  DTDDVTYQVY  EEQAVYEPLE  NEGIEITEVT  APPEDNPVED  SQVIVEEVSI  301
FPVEEQQEVP  PETNRKTDDP  EQKAKVKKKK  PKLLNKFDKT  IKAELDAAEK  LRKRGKIEEA  361
VNAFKELVRK  YPQSPRARYG  KAQCEDDLAE  KRRSNEVLRG  AIETYQEVAS  LPDVPADLLK  421
LSLKRRSDRQ  QFLGHMRGSL  LTLQRLVQLF  PNDTSLKNDL  GVGYLLIGDN  DNAKKVYEEV  481
LSVTPNDGFA  KVHYGFILKA  QNKIAESIPY  LKEGIESGDP  GTDDGRFYFH  LGDAMQRVGN  541
KEAYKWYELG  HKRGHFASVW  QRSLYNVNGL  KAQPWWTPKE  TGYTELVKSL  ERNWKLIRDE  601
GLAVMDKAKG  LFLPEDENLR  EKGDWSQFTL  WQQGRRNENA  CKGAPKTCTL  LEKFPETTGC  661
RRGQIKYSIM  HPGTHVWPHT  GPTNCRLRMH  LGLVIPKEGC  KIRCANETRT  WEEGKVLIFD  721
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DSFEHEVWQD ASSFRLIFIV DVWHPELTPQ QRRSLPAI (SEQ ID NO:2; GENBANK
Accession No. S83325; His motif is underlined; conserved
sequences within the catalytic domain are designated by bold
type)

5   For example, a compound which inhibits HAAH
hydroxylation is a polypeptide that binds a HAAH ligand but
does not transduce an intracellular signal or an polypeptide
which contains a mutation in the catalytic site of HAAH.
Such a polypeptide contains an amino acid sequence that is

10   at least 50% identical to a naturally-occurring HAAH amino
acid sequence or a fragment thereof and which has the
ability to inhibit HAAH hydroxylation of substrates
containing an EGF-like repeat sequence. More preferably,
the polypeptide contains an amino acid sequence that is at

15   least 75%, more preferably at least 85%, more preferably at
least 95% identical to SEQ ID NO: .

A substantially pure HAAH polypeptide or HAAH-
derived polypeptide such as a mutated HAAH polypeptide is
preferably obtained by expression of a recombinant nucleic

20   acid encoding the polypeptide or by chemically synthesizing
the protein. A polypeptide or protein is substantially pure
when it is separated from those contaminants which accompany
it in its natural state (proteins and other naturally-
occurring organic molecules). Typically, the polypeptide is

25   substantially pure when it constitutes at least 60%, by
weight, of the protein in the preparation. Preferably, the
protein in the preparation is at least 75%, more preferably
at least 90%, and most preferably at least 99%, by weight,
HAAH. Purity is measured by any appropriate method, e.g.,

30   column chromatography, polyacrylamide gel electrophoresis,
or HPLC analysis. Accordingly, substantially pure
polypeptides include recombinant polypeptides derived from a
eucaryote but produced in *E. coli* or another procaryote, or

in a eucaryote other than that from which the polypeptide
was originally derived.

Nucleic acid molecules which encode such HAAH or
HAAH-derived polypeptides are also within the invention.

Table 2:   HAAH cDNA sequence

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cggaccgtgc aatggcccag cgtaagaatg ccaagagcag cggcaacagc agcagcagcg 61
gctccggcag cggtagcacg agtgcgggca gcagcagccc cggggcccgg agagagacaa 121
agcatggagg acacaagaat gggaggaaag gcggactctc gggaacttca ttcttcacgt 181
ggtttatggt gattgcattg ctgggcgtct ggacatctgt agctgtcgtt tggtttgatc 241
ttgttgacta tgaggaagtt ctaggaaaac taggaatcta tgatgctgat ggtgatggag 301
attttgatgt ggatgatgcc aaagttttat taggacttaa agagagatct acttcagagc 361
cagcagtccc gccagaagag gctgagccac acactgagcc cgaggagcag gttcctgtgg 421
aggcagaacc ccagaatatc gaagatgaag caaaagaaca aattcagtcc cttctccatg 481
aaatggtaca cgcagaacat gttgagggag aagacttgca acaagaagat ggacccacag 541
gagaaccaca acaagaggat gatgagtttc ttatggcgac tgatgtagat gatagatttg 601
agaccctgga acctgaagta tctcatgaag aaaccgagca tagttaccac gtggaagaga 661
cagtttcaca agactgtaat caggatatgg aagagatgat gtctgagcag gaaaatccag 721
attccagtga accagtagta gaagatgaaa gattgcacca tgatacagat gatgtaacat 781
accaagtcta tgaggaacaa gcagtatatg aacctctaga aaatgaaggg atagaaatca 841
cagaagtaac tgctccccct gaggataatc ctgtagaaga ttcacaggta attgtagaag 901
aagtaagcat ttttcctgtg gaagaacagc aggaagtacc accagaaaca aatagaaaaa 961
cagatgatcc agaacaaaaa gcaaaagtta agaaaaagaa gcctaaactt ttaaataaat 1021
ttgataagac tattaaagct gaacttgatg ctgcagaaaa actccgtaaa aggggaaaaa 1081
ttgaggaagc agtgaatgca tttaaagaac tagtacgcaa ataccctcag agtccacgag 1141
caagatatgg gaaggcgcag tgtgaggatg atttggctga gaagaggaga agtaatgagg 1201
tgctacgtgg agccatcgag acctaccaag aggtggccag cctacctgat gtccctgcag 1261
acctgctgaa gctgagtttg aagcgtcgct cagacaggca acaatttcta ggtcatatga 1321
gaggttccct gcttaccctg cagagattag ttcaactatt tcccaatgat acttccttaa 1381
aaaatgacct tggcgtggga tacctcttga taggagataa tgacaatgca aagaaagttt 1441
atgaagaggt gctggatggtg acacctaatg atggcttttgc taaagtccat tatggcttca 1501
tcctgaaggc acagaacaaa attgctgaga gcatcccata tttaaaggaa ggaatagaat 1561
ccggagatcc tggcactgat gatgggagat tttatttcca cctgggggat gccatgcaga 1621
gggttgggaa caaagaggca tataagtggt atgagcttgg gcacaagaga ggacactttg 1681
catctgtctg gcaacgctca ctctacaatg tgaatggact gaaagcacag ccttggtgga 1741
ccccaaaaga aacgggctac acagagttag taaagtcttt agaaagaaac tggaagttaa 1801
tccgagatga aggccttgca gtgatggata aagccaaagg tctcttcctg cctgaggatg 1861
aaaacctgag ggaaaaaggg gactggagcc agttcacgct gtggcagcaa ggaagaagaa 1921
atgaaaatgc ctgcaaagga gctcctaaaa cctgtacctt actagaaaag ttccccgaga 1981
caacaggatg cagaagagga cagatcaaat attccatcat gcaccccggg actcacgtgt 2041
ggccgcacac agggcccaca aactgcaggc tccgaatgca cctgggcttg gtgattccca 2101
aggaaggctg caagattcga tgtgccaacg agaccaggac ctgggaggaa ggcaaggtgc 2161
tcatctttga tgactccttt gagcacgagg tatggcagga tgcctcatct ttccggctga 2221
tattcatcgt ggatgtgtgg catccggaac tgacaccaca gcagagacgc agccttccag 2281
caatttagca tgaattcatg caagcttggg aaactctgga gaga
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(SEQ ID NO:3 ; GENBANK Accession No. S83325; codon encoding
initiating methionine is underlined).

Methods of inhibiting tumor growth also include
administering a compound which inhibits HAAH hydroxylation
of a NOTCH polypeptide.   For example, the compound inhibits
hydroxylation of an EGF-like cysteine-rich repeat sequence
in a NOTCH polypeptide, e.g., one containing the consensus

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sequence CDXXXCXXKXGNGXCDXXCNNAACXXDGXDC (SEQ ID NO:4).
Polypeptides containing an EGF-like cysteine-rich repeat
sequence are administered to block hydroxylation of
endogenous NOTCH.

5   Growth of a tumor which overexpresses HAAH is also
inhibited by administering a compound which inhibits signal
transduction through the insulin receptor substrate (IRS)
signal transduction pathway. Preferably the compound
inhibits IRS phosphorylation. For example, the compound is
10   a peptide or non-peptide compound which binds to and
inhibits phosphorylation at residues 46, 465, 551, 612, 632,
662, 732, 941, 989, or 1012 of SEQ ID NO:5 . Compounds
include polypeptides such those which block an IRS
phosphorylation site such as a Glu/Tyr site. Antibodies
15   such as those which bind to a carboxyterminal domain of IRS
containing a phosphorylation site block IRS phosphorylation,
and as a consequence, signal transduction along the pathway.
Inhibition of IRS phosphorylation in turn leads to
inhibition of cell proliferation. Other compounds which
20   inhibit IRS phosphorylation include vitamin D analogue
EB1089 and Wortmannin.

        HAAH-overproducing tumor cells were shown to express
HAAH both intracellularly and on the surface of the tumor
cell. Accordingly, a method of killing a tumor cell is
25   carried out by contacting such a tumor cell with a cytotoxic
agent linked to an HAAH-specific antibody. The HAAH-
specific antibody (antibody fragment, or ligand which binds
to extracellular HAAH) directs the chimeric polypeptide to
the surface of the tumor cell allowing the cytotoxic agent
30   to damage or kill the tumor cell to which the antibody is
bound. The monoclonal antibody binds to an epitope of HAAH
such as an epitope exposed on the surface of the cell or in

the catalytic site of HAAH. The cytotoxic composition
preferentially kills tumor cells compared to non-tumor cell.

Screening methods to identify anti-tumor agents
which inhibit the growth of tumors which overexpress HAAH
5   are also within the invention. A screening method used to
determine whether a candidate compound inhibits HAAH
enzymatic activity includes the following steps: (a)
providing a HAAH polypeptide, e.g., a polypeptide which
contains the carboxyterminal catalytic site of HAAH; (b)
10 providing a polypeptide comprising an EGF-like domain; (c)
contacting the HAAH polypeptide or the EGF-like polypeptide
with the candidate compound; and (d) determining
hydroxylation of the EGF-like polypeptide of step (b). A
decrease in hydroxylation in the presence of the candidate
15 compound compared to that in the absence of said compound
indicates that the compound inhibits HAAH hydroxylation of
EGF-like domains in proteins such as NOTCH.

Anti-tumor agents which inhibit HAAH activation of
NOTCH are identified by (a) providing a cell expressing
20 HAAH; (b) contacting the cell with a candidate compound; and
(c) measuring translocation of activated NOTCH to the
nucleus of said cell. Translocation is measured by using a
reagent such as an antibody which binds to a 110 kDa
activation fragment of NOTCH. A decrease in translocation
25 in the presence of the candidate compound compared to that
in the absence of the compound indicates that the compound
inhibits HAAH activation of NOTCH, thereby inhibiting NOTCH-
mediated signal transduction and proliferation of HAAH-
overexpressing tumor cells.

30 Nucleotide and amino acid comparisons described
herein were carried out using the Lasergene software package
(DNASTAR, Inc., Madison, WI). The MegAlign module used was
the Clustal V method (Higgins et al., 1989, CABIOS 5(2):151-

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153). The parameter used were gap penalty 10, gap length penalty 10.

Hybridization is carried out using standard techniques, such as those described in Ausubel *et al.*
(*Current Protocols in Molecular Biology*, John Wiley & Sons, 1989). "High stringency" refers to nucleic acid hybridization and wash conditions characterized by high temperature and low salt concentration, *e.g.*, wash conditions of 65°C at a salt concentration of 0.1 X SSC. "Low" to "moderate" stringency refers to DNA hybridization and wash conditions characterized by low temperature and high salt concentration, *e.g.*, wash conditions of less than 60°C at a salt concentration of at least 1.0 X SSC. For example, high stringency conditions include hybridization at 42°C in the presence of 50% formamide; a first wash at 65°C in the presence of 2 X SSC and 1% SDS; followed by a second wash at 65°C in the presence of 0.1% x SSC. Lower stringency conditions suitable for detecting DNA sequences having about 50% sequence identity to an HAAH gene sequence are detected by, for example, hybridization at about 42°C in the absence of formamide; a first wash at 42°C, 6 X SSC, and 1% SDS; and a second wash at 50°C, 6 X SSC, and 1% SDS.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Brief Description of the Drawings

Fig. 1 is a bar graph showing colony formation induced by transient transfection of NIH-3T3 cells with various AAH cDNAs. Colony formation was induced by transient transfection with 10 μg DNA. In contrast, the mutant murine AAH construct without enzymatic activity has no transforming activity. The data is presented as mean number of transformed foci ± SEM.

Fig. 2 is a bar graph showing the results of a densitometric analysis of a Western blot assay of proteins produced by various murine AAH stably transfected cell clones. In clones 7 and 18, there was a modest increase in

5 HAAH gene expression, while the overexpression was to a lesser degree in clone 16.

Figs. 3A-B are bar graphs showing colony formation in soft agar exhibited by HAAH stably transfected clones compared to HAAH enzymatic activity. Fig. 3A shows a

10 measurement of murine AAH enzymatic activity in clones 7, 16 and 18, and Fig. 3B shows colony formation exhibited by clones 7, 16 and 18. Data is presented as mean number of colonies 10 days after plating ± SEM. All three clones with modest increases in HAAH enzymatic activity, that correlated

15 with protein expression, exhibited anchorage independent growth.

Fig. 4 is a bar graph showing tumor formation in nude mice injected with transfected clones overexpressing murine AAH. Tumor growth was assessed after 30 days. Mean

20 tumor weight observed in mice injected with clones 7, 16 and 18 as compared to mock DNA transfected clone. All animals injected with clones overexpressing HAAH developed tumors.

Figs. 5A-D are bar graphs showing increased AAH expression in PNET2 (Fig. 5A, 5C) and SH-Sy5y (Fig. 5B)

25 cells treated with retinoic acid (Figs. 5A, 5B) or phorbol ester myristate (PMA; Fig. 5C) to induce neurite outgrowth as occurs during tumor cell invasion. The cells were treated with 10 μM retinoic acid or 100 nM PMA for 0, 1, 2, 3, 4, or 7 days. Cell lysates were analyzed by Western blot

30 analysis using an HAAH-specific monoclonal antibody to detect the 85 kDa AAH protein. The levels of immunoreactivity were measured by volume densitometry (arbitrary units). The graphs indicate the mean ± S.D. of

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results obtained from three separate experiments. In Fig. 5D, PNET2 cells were treated for 24 hours with sub-lethal concentrations of H_2O_2 to induce neurite retraction. Viability of greater than 90% of the cells was demonstrated by Trypan blue dye exclusion. Similar results were obtained for SH-Sy5y cells.

Fig. 6 is a bar graph showing the effects of AAH over-expression on the levels of anti-apoptosis (Bcl-2), cell cycle-mitotic inhibitor (p16 and p21/Waf1), and proliferation (proliferating cell nuclear antigen; PCNA) molecules. PNET2 neuronal cells were stably transfected with the full-length human cDNA encoding AAH (pHAAH) or empty vector (pcDNA). AAH gene expression was under control of a CMV promoter. Western blot analysis was performed with cell lysates prepared from cultures that were 70 to 80 percent confluent. Protein loading was equivalent in each lane. Replicate blots were probed with the different antibodies. Bar graphs depict the mean S.D.'s of protein expression levels measured in three experiments. All differences are statistically significant by Student T-test analysis (P<0.01-P<0.001).

Fig. 7 is a diagram of showing the components of the IRS-1 signal transduction pathway.

Fig. 8 is a line graph showing growth curves generated in cells expressing the antisense HAAH compared to controls expressing GFP.

Fig. 9 is a diagram of the functional domains of the hIRS-1 protein and structural organization of the point mutants. All mutant and "wild type" hIRS-1 proteins construct contain a FLAG (F) epitope (DYKDDDDK; SEQ ID NO:7) at the C-terminus. PH and PTB indicate pleckstrin homology and phosphotyrosine binding, regions, respectively.

Detailed Description

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HAAH is a protein belonging to the (α-ketoglutarate dependent dioxygenase family of prolyl and lysyl hydroxylases which play a key role in collagen biosynthesis. This molecule hydroxylates aspartic acid or asparagine residues in EGF-like domains of several proteins in the presence of ferrous iron. These EGF-like domains contain conserved motifs, that form repetitive sequences in proteins such as clotting factors, extracellular matrix proteins, LDL receptor, NOTCH homologues or NOTCH ligand homologues.

The alpha-ketoglutarate-dependent dioxygenase aspartyl (asparaginyl) beta-hydroxylase (AAH) specifically hydroxylates one aspartic or asparagine residue in EGF-like domains of various proteins. The 4.3-kb cDNA encoding the human AspH (hAspH) hybridizes with 2.6 kb and 4.3 kb transcripts in transformed cells, and the deduced amino.acid sequence of the larger transcript encodes an protein of about 85 kDa. Both *in vitro* transcription and translation and Western blot analysis also demonstrate a 56-kDa protein that may result from posttranslational cleavage of the catalytic C terminus.

An physiological function of AAH is the post-translational beta-hydroxylation of aspartic acid in vitamin K-dependent coagulation proteins. However, the abundant expression of AAH in several malignant neoplasms, and low levels of AAH in many normal cells indicate a role for this enzyme in malignancy. The AAH gene is also highly expressed in cytotrophoblasts, but not syncytiotrophoblasts of the placenta. Cytotrophoblasts are invasive cells that mediate placental implantation. The increased levels of AAH expression in human cholangiocarcinomas, hepatocellular carcinomas, colon cancers, and breast carcinomas were primarily associated with invasive or metastatic lesions. Moreover, overexpression of AAH does not strictly reflect

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increased DNA synthesis and cellular proliferation since
high levels of AAH immunoreactivity were observed in
100 percent of cholangiocarcinomas, but not in human or
experimental disease processes associated with regeneration
5   or nonneoplastic proliferation of bile ducts. AAH
overexpression and attendant high levels of beta hydroxylase
activity lead to invasive growth of transformed neoplastic
cells. Detection of an increase in HAAH expression is
useful for early and reliable diagnosis of the cancer types
10   which have now been characterized as overexpressing this
gene product.

Diagnosis of malignant tumors

HAAH is overexpressed in many tumors of endodermal
origin and in at least 95% of CNS tumors compared to normal
15   noncancerous cells. An increase in HAAH gene product in a
patient-derived tissue sample (e.g., solid tissue or bodily
fluid) is carried out using standard methods, e.g., by
Western blot assays or a quantitative assay such as ELISA.
For example, a standard competitive ELISA format using an
20   HAAH-specific antibody is used to quantify patient HAAH
levels. Alternatively, a sandwich ELISA using a first
antibody as the capture antibody and a second HAAH-specific
antibody as a detection antibody is used.

Methods of detecting HAAH include contacting a
25   component of a bodily fluid with an HAAH-specific antibody
bound to solid matrix, e.g., microtiter plate, bead,
dipstick. For example, the solid matrix is dipped into a
patient-derived sample of a bodily fluid, washed, and the
solid matrix is contacted with a reagent to detect the
30   presence of immune complexes present on the solid matrix.

Proteins in a test sample are immobilized on (bound
to) a solid matrix. Methods and means for covalently or
noncovalently binding proteins to solid matrices are known

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in the art. The nature of the solid surface may vary depending upon the assay format. For assays carried out in microtiter wells, the solid surface is the wall of the well or cup. For assays using beads, the solid surface is the

5   surface of the bead. In assays using a dipstick (i.e., a solid body made from a porous or fibrous material such as fabric or paper) the surface is the surface of the material from which the dipstick is made. Examples of useful solid supports include nitrocellulose (e.g., in membrane or

10   microtiter well form), polyvinyl chloride (e.g., in sheets or microtiter wells), polystyrene latex (e.g., in beads or microtiter plates, polyvinylidine fluoride (known as IMMULON™), diazotized paper, nylon membranes, activated beads, and Protein A beads. The solid support containing

15   the antibody is typically washed after contacting it with the test sample, and prior to detection of bound immune complexes. Incubation of the antibody with the test sample is followed by detection of immune complexes by a detectable label. For example, the label is enzymatic, fluorescent,

20   chemiluminescent, radioactive, or a dye. Assays which amplify the signals from the immune complex are also known in the art, e.g., assays which utilize biotin and avidin.

An HAAH-detection reagent, e.g., an antibody, is packaged in the form of a kit, which contains one or more

25   HAAH-specific antibodies, control formulations (positive and/or negative), and/or a detectable label. The assay may be in the form of a standard two-antibody sandwich assay format known in the art.

Production of HAAH-specific antibodies

30   Anti-HAAH antibodies were obtained by techniques well known in the art. Such antibodies are polyclonal or monoclonal. Polyclonal antibodies were obtained, for example, by the methods described in Ghose et al., Methods

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in Enzymology, Vol. 93, 326-327, 1983. An HAAH polypeptide, or an antigenic fragment thereof, was used as the immunogen to stimulate the production of polyclonal antibodies in the antisera of rabbits, goats, sheep, or rodents. Antigenic

5   polypeptides for production of both polyclonal and monoclonal antibodies useful as immunogens include polypeptides which contain an HAAH catalytic domain. For example, the immunogenic polypeptide is the full-length mature HAAH protein or an HAAH fragment containing the

10   carboxyterminal catalytic domain e.g., an HAAH polypeptide containing the His motif of SEQ ID NO:2.

Antibodies which bind to the same epitopes as those antibodies disclosed herein as identified using standard methods, e.g., competitive binding assays, known in the art.

15   Monoclonal antibodies were obtained by standard techniques. Ten μg of purified recombinant HAAH polypeptide was administered to mice intraperitoneally in complete Freund's adjuvant, followed by a single boost intravenously (into the tail vein) 3-5 months after the initial

20   inoculation. Antibody-producing hybridomas were made using standard methods. To identify those hybridomas producing antibodies that are highly specific for an HAAH polypeptide, hybridomas were screened using the same polypeptide immunogen used to immunize. Those antibodies which were

25   identified as having HAAH-binding activity are also screened for the ability to inhibit HAAH catalytic activity using the enzymatic assays described below. Preferably, the antibody has a binding affinity of at least about 10^8 liters/mole and more preferably, an affinity of at least about 10^9

30   liters/mole.

Monoclonal antibodies are humanized by methods known in the art, e.g, MAbs with a desired binding specificity can

be commercially humanized (Scotgene, Scotland; Oxford
Molecular, Palo Alto, CA).

HAAH-specific intrabodies are produced as follows.
Following identification of a hybridoma producing a suitable
5   monoclonal antibody, DNA encoding the antibody is cloned.
DNA encoding a single chain HAAH-specific antibody in which
heavy and light chain variable domains are separated by a
flexible linker peptide is cloned into an expression vector
using known methods (e.g., Marasco et al., 1993, Proc.
10   Natl. Acad. Sci. USA 90:7889-7893 and Marasco et al.,
1997, Gene Therapy 4:11-15). Such constructs are introduced
into cells, e.g., using standard gene delivery techniques
for intracellular production of the antibodies.
Intracellular antibodies, i.e., intrabodies, are used to
15   inhibit signal transduction by HAAH. Intrabodies which bind
to a carboxyterminal catalytic domain of HAAH inhibit the
ability of HAAH to hydroxylate EGF-like target sequences.

Methods of linking HAAH-specific antibodies (or
fragments thereof) which bind to cell surface exposed
20   epitopes of HAAH on the surface of a tumor cell are linked
to known cytotoxic agents, e.g, ricin or diptheria toxin,
using known methods.

Methods of treating malignant tumors

Patients with tumors characterized as overexpressing
25   HAAH as such tumors of endodermal origin or CNS tumors are
treated by administering HAAH antisense nucleic acids.

Antisense therapy is used to inhibit expression of
HAAH in patients suffering from hepatocellular carcinomas,
cholangiocarcinomas, glioblastomas and neuroblastomas. For
30   example, an HAAH antisense strand (either RNA or DNA) is
directly introduced into the cells in a form that is capable
of binding to the mRNA transcripts. Alternatively, a vector
containing a sequence which, which once within the target

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cells, is transcribed into the appropriate antisense mRNA, may be administered. Antisense nucleic acids which hybridize to target mRNA decrease or inhibit production of the polypeptide product encoded by a gene by associating

5   with the normally single-stranded mRNA transcript, thereby interfering with translation and thus, expression of the protein.   For example, DNA containing a promoter, e.g., a tissue-specific or tumor specific promoter, is operably linked to a DNA sequence (an antisense template), which is

10   transcribed into an antisense RNA. By "operably linked" is meant that a coding sequence and a regulatory sequence(s) (*i.e.*, a promoter) are connected in such a way as to permit gene expression when the appropriate molecules (*e.g.*, transcriptional activator proteins) are bound to the

15   regulatory sequence(s).

Oligonucleotides complementary to various portions of HAAH mRNA are tested *in vitro* for their ability to decrease production of HAAH in tumor cells (e.g., using the FOCUS hepatocellular carcinoma (HCC) cell line) according to

20   standard methods.   A reduction in HAAH gene product in cells contacted with the candidate antisense composition compared to cells cultured in the absence of the candidate composition is detected using HAAH-specific antibodies or other detection strategies.   Sequences which decrease

25   production of HAAH in *in vitro* cell-based or cell-free assays are then be tested *in vivo* in rats or mice to confirm decreased HAAH production in animals with malignant neoplasms.

Antisense therapy is carried out by administering to

30   a patient an antisense nucleic acid by standard vectors and/or gene delivery systems.   Suitable gene delivery systems may include liposomes, receptor-mediated delivery systems, naked DNA, and viral vectors such as herpes

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viruses, retroviruses, adenoviruses and adeno-associated
viruses, among others. A reduction in HAAH production
results in a decrease in signal transduction via the IRS
signal transduction pathway. A therapeutic nucleic acid
5   composition is formulated in a pharmaceutically acceptable
carrier. The therapeutic composition may also include a
gene delivery system as described above. Pharmaceutically
acceptable carriers are biologically compatible vehicles
which are suitable for administration to an animal: e.g.,
10   physiological saline. A therapeutically effective amount of
a compound is an amount which is capable of producing a
medically desirable result such as reduced production of an
HAAH gene product or a reduction in tumor growth in a
treated animal.

15   Parenteral administration, such as intravenous,
subcutaneous, intramuscular, and intraperitoneal delivery
routes, may be used to deliver nucleic acids or HAAH-
inhibitory peptides or non-peptide compounds. For treatment
of CNS tumors, direct infusion into cerebrospinal fluid is
20   useful. The blood-brain barrier may be compromised in
cancer patients, allowing systemically administered drugs to
pass through the barrier into the CNS. Liposome
formulations of therapeutic compounds may also facilitate
passage across the blood-brain barrier.

25   Dosages for any one patient depends upon many
factors, including the patient's size, body surface area,
age, the particular nucleic acid to be administered, sex,
time and route of administration, general health, and other
drugs being administered concurrently. Dosage for _
30   intravenous administration of nucleic acids is from
approximately 10^6 to 10^{22} copies of the nucleic acid
molecule.

Ribozyme therapy is also be used to inhibit HAAH gene expression in cancer patients. Ribozymes bind to specific mRNA and then cut it at a predetermined cleavage point, thereby destroying the transcript. These RNA molecules are used to inhibit expression of the HAAH gene according to methods known in the art (Sullivan et al., 1994, J. Invest. Derm. 103:85S-89S; Czubayko et al., 1994, J. Biol. Chem. 269:21358-21363; Mahieu et al, 1994, Blood 84:3758-65; Kobayashi et al. 1994, Cancer Res. 54:1271-1275).

Methods of identifying compounds that inhibit HAAH enzymatic activity

Aspartyl (asparaginyl) beta-hydroxylaseydroxylase (AAH) activity is measured in vitro or in vivo. For example, HAAH catalyzes posttranslational modification of β carbon of aspartyl and asparaginyl residues of EGF-like polypeptide domains. An assay to identify compounds which inhibit hydroxylase activity is carried out by comparing the level of hydroxylation in an enzymatic reaction in which the candidate compound is present compared to a parallel reaction in the absence of the compound (or a predetermined control value). Standard hydroxylase assays carried out in a testtube are known in the art, e.g., Lavaissiere et al., 1996, J. Clin. Invest. 98:1313-1323; Jia et al., 1992, J. Biol. Chem. 267:14322-14327; Wang et al., 1991, J. Biol. Chem. 266:14004-14010; or Gronke et al., 1990, J. Biol. Chem. 265:8558-8565. Hydroxylase activity is also measured using carbon dioxide ($^{14}CO_2$ capture assay) in a 96-well microtiter plate format (Zhang et al., 1999, Anal. Biochem. 271:137-142. These assays are readily automated and suitable for high throughput screening of candidate compounds to identify those with hydroxylase inhibitory activity.

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Candidate compound which inhibit HAAH activation of NOTCH are identified by detecting a reduction in activated NOTCH in a cell which expresses or overexpresses HAAH, e.g., FOCUS HCC cells. The cells are cultured in the presence of a candidate compound. Parallel cultures are incubated in the absence of the candidate compound. To evaluate whether the compound inhibits HAAH activation of NOTCH, translocation of activated NOTCH to the nucleus of the cell is measured. Translocation is measured by detecting a 110 kDa activation fragment of NOTCH in the nucleus of the cell. The activation fragment is cleaved from the large (approximately 300 kDa) transmembrane NOTCH protein upon activation. Methods of measuring NOTCH translocation are known, e.g, those described by Song et al., 1999, Proc. Natl. Acad. Sci U.S.A. 96:6959-6963 or Capobianco et al., 1997, Mol. Cell Biol. 17:6265-6273. A decrease in translocation in the presence of the candidate compound compared to that in the absence of the compound indicates that the compound inhibits HAAH activation of NOTCH, thereby inhibiting NOTCH-mediated signal transduction and proliferation of HAAH-overexpressing tumor cells.

Methods of screening for compounds which inhibit phosphorylation of IRS are carried out by incubating IRS-expressing cells in the presence and absence of a candidate compound and evaluating the level of IRS phosphorylation in the cells. A decrease in phosphorylation in cells cultured in the presence of the compound compared to in the absence of the compound indicates that the compound inhibits IRS-1 phosphorylation, and as a result, growth of HAAH-overexpressing tumors. Alternatively, such compounds are identified in an *in vitro* phosphorylation assay known in the art, e.g., one which measured phosphorylation of a synthetic substrate such as poly (Glu/Tyr).

Example 1: Increased expression of HAAH is associated with malignant transformation

HAAH is a highly conserved enzyme that hydroxylates EGF-like domains in transformation associated proteins. The HAAH gene is overexpressed in human hepatocellular carcinomas and cholangiocarcinomas. HAAH gene expression was found to be undetectable during bile duct proliferation in both human disease and rat models compared to cholangiocarcinoma. Overexpression of HAAH in NIH-3T3 cells was associated with generation of a malignant phenotype, and enzymatic activity was found to be required for cellular transformation. The data described below indicate that overexpression of HAAH is linked to cellular transformation of biliary epithelial cells.

To identify molecules that are specifically overexpressed in transformed malignant cells of human hepatocyte origin, the FOCUS hepatocellular carcinoma (HCC) cell line was used as an immunogen to generate monoclonal antibodies (mAb) that specifically or preferentially recognize proteins associated with the malignant phenotype. A lambda GT11 cDNA expression library derived from HepG2 HCC cells was screened, and HAAH-specific mAb produced against the FOCUS cell line was found to recognize an epitope on a protein encoded by an HAAH cDNA. The HAAH enzyme was found to be upregulated in several different human transformed cell lines and tumor tissues compared to adjacent human tissue counterparts. The overexpressed HAAH enzyme in different human malignant tissues was found to be catalytically active.

HAAH gene expression was examined in proliferating bile ducts and in NIH 3T3 cells. Its role in the generation of the malignant phenotype was measured by the formation of transformed foci, growth in soft agar as an index of

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anchorage independent growth and tumor formation in nude mice. The role of enzymatic activity in the induction of transformed phenotype was measured by using a cDNA construct with a mutation in the catalytic site that abolished

5 hydroxylase activity. The results indicated that an increase in expression of HAAH gene is associated with malignant transformation of bile ducts.

The following materials and methods were used to generate the data described below.

10 Antibodies

The FB50 monoclonal antibody was generated by cellular immunization of Balb/C mice with FOCUS HCC cells. A monoclonal anti-Dengue virus antibody was used as a non-relevant control. The HBOH2 monoclonal antibody was

15 generated against a 52 kDa recombinant HAAH polypeptide and recognizes the catalytic domain of beta-hydroxylase from mouse and human proteins. Polyclonal anti-HAAH antibodies cross-react with rat hydroxylase protein. Control antibody anti-Erk-1 was purchased from Santa Cruz Biotechnology, Inc,

20 CA. Sheep anti-mouse and donkey anti-rabbit antisera labeled with horseradish peroxidase were obtained from Amersham, Arlington Heights, IL.

Constructs

The murine full length AAH construct (pNH376) and

25 the site-directed mutation construct (pNH376-H660) with abolished catalytic activity were cloned into the eukaryotic expression vector pcDNA3 (Invitrogen Corp., San Diego, CA). The full length human AAH was cloned into prokaryotic expression vector pBC-SK+ (Stratagene, La Jolla, CA). The

30 full length human AAH (GENBANK Accession No. S83325) was subcloned into the EcoRI site of the pcDNA3 vector.

Animal model of bile duct proliferation

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Rats were divided into 9 separate groups of 3 animals each except for group 9 which contained 5 rats. Group 1 was the non-surgical control group, and group 2 was the sham-operated surgical control. The remaining groups underwent common bile duct ligation to induce intrahepatic bile duct proliferation and were evaluated at 6, 12, 24, 48 hours and 4, 8 and 16 days as shown in Table 3. Animals were asphyxiated with CO_2, and liver samples were taken from left lateral and median lobes, fixed in 2 % paraformaldehyde and embedded in paraffin. Liver samples (5 μm) were cut and stained with hematoxylin and eosin to evaluate intrahepatic bile duct proliferation. Immunohistochemistry was performed with polyclonal anti-HAAH antibodies that cross-react with the rat protein to determine levels of protein expression.

Bile duct proliferation associated with primary sclerosing cholangitis (PSC)

Liver biopsy samples were obtained from 7 individuals with PSC and associated bile duct proliferation. These individuals were evaluated according to standard gastroenterohepatological protocols. Patients were 22-46 years of age and consisted of 4 males and 3 females. Four had associated inflammatory bowel disease (3 ulcerative colitis and 1 Crohn's colitis). All patients underwent a radiological evaluation including abdominal ultrasonography and endoscopic retrograde cholangiopancreaticography to exclude the diagnosis of extrahepatic biliary obstruction. Tissue sections were prepared from paraffin embedded blocks and were evaluated by hematoxylin and eosin staining for bile duct proliferation. Expression of HAAH was determined by immunohistochemistry using an HAAH-specific monoclonal antibody such as FB50.

Immunohistochemistry

Liver tissue sections (5 μm) were deparaffinized in xylene and rehydrated in graded alcohol. Endogenous peroxidase activity was quenched by a 30-minute treatment with 0.6 % H_2O_2 in 60% methanol. Endogenous biotin was masked by incubation with avidin-biotin blocking solutions (Vector Laboratories, Burlingame, CA). The FB50 mAb (for PSC samples) and polyclonal anti-HAAH-hydroxylase antibodies (for rat liver samples) were added to slides in a humidified chamber at 4°C overnight. Immunohistochemical staining was performed using a standard avidin-biotin horseradish peroxidase complex (ABC) method using Vectastain Kits with diaminobenzidine (DAB) as the chromogen according to manufacturer's instructions (Vector Laboratories, Inc., Burlingame, CA). Tissue sections were counterstained with hematoxylin, followed by dehydration in ethanol. Sections were examined by a light microscopy for bile duct proliferation and HAAH protein expression. Paraffin sections of cholangiocarcinoma and placenta were used as positive controls, and hepatosteatosis samples were used as a negative controls. To control for antibody binding specificity, adjacent sections were immunostained in the absence of a primary antibody, or using non-relevant antibody to Dengue virus. As a positive control for tissue immunoreactivity, adjacent sections of all specimens were immunostained with monoclonal antibody to glyceraldehyde 3-phosphate dehydrogenase.

Western blot analysis

Cell lysates were prepared in a standard radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors. The total amount of protein in the lysates was determined by Bio-Rad colorimetric assay (Bio Rad, Hercules, CA) followed by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE),

transferred to PVDF membranes, and subjected to Western blot analysis using FB50, HBOH2, anti-Erk-1 (used as an internal control for protein loading) as primary, sheep anti-mouse and donkey anti-rabbit antisera labeled with horseradish

5   peroxidase as secondary antibodies. Antibody binding was detected with enhanced chemiluminescence reagents (SuperSignal, Pierce Chemical Company, Rockford, IL) and film autoradiography. The levels of immunoreactivity were measured by volume densitometry using NIH Image software.

10   ### Enzymatic activity assay

AAH activity was measured in cell lysates using the first EGF-like domain of bovine protein S as substrate where ^{14}C-labeled α-ketogluterate hydroxylates the domain releasing ^{14}C containing CO_2 according to standard methods,

15   e.g., those described by Jia et al., 1992, J. Biol. Chem. 267:14322-14327; Wang et al., 1991, J. Biol. Chem. 266:14004-14010; or Gronke et al., 1990, J. Biol. Chem. 265:8558-8565. Incubations were carried out at 37°C for 30 min in a final volume of 40 μl containing 48 μg of crude

20   cell extract protein and 75 μM EGF substrate.

Cell transfection studies

The NIH-3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Washington, DC) supplemented with 10 % heat-inactivated fetal calf serum

25   (FCS; Sigma Chemical Co., St.Louis, MO), 1% L-glutamine, 1% non-essential amino acids and 1% penicillin-streptomycin (GIBCO BRL, Life Technologies, Inc., Grand Island, NY). Subconfluent NIH-3T3 cells (3 x 10^5 cells/60-mm dish) were transfected with 10 μg of one of the following plasmids:

30   1) non-recombinant pcDNA3 vector (Invitrogen Corp., San Diego, CA) as a negative control; 2) pNH376-H660, the murine AAH cDNA that was mutated in the catalytic domain and cloned into the pcDNA3 vector driven by a CMV promoter; 3) pNH376,

the wild type murine AAH cDNA cloned into the pcDNA3 vector;
4) pCDHH, wild type human AAH cDNA cloned into the pcDNA3
vector; or 5) pLNCX-UP1, a cDNA that encodes v-Src oncogene
(positive control). Cells were transfected using the
calcium phosphate transfection kit according to
manufacturer's instructions (5 Prime - 3 Prime, Inc.,
Boulder, CO). Comparison of cellular transfection
efficiency was assessed with the various constructs. For
this procedure, confluent plates obtained 48 hours after
transfection were split and reseeded into 12 separate 6-cm
dishes, and 6 of them were made to grow in the presence of
400 μg/ml G-418 (GIBCO BRL, Life Technologies, Inc., Grant
Island, NY) containing medium. The number of G-418
resistant foci was determined at 14 days after transfection
and used to correct for any variability in transfection
efficiency.

Transformation assay

The NIH-3T3 cells were transfected with the various
constructs and allowed to reach confluence after 48 hours as
described above. Each 6 cm dish was split and seeded into
12 different 6 cm dishes. While 6 of them were made to grow
in the presence of G-418 to detect transfection efficiency,
the other six were grown in complete medium without G-418
and with a medium change every 4th day. The number of
transformed foci were counted in these plates without G-418
and expressed as transformed foci per μg transfected DNA.

Anchorage-independent cell growth assay

A limiting dilution technique (0.15 cell/well of a
flat bottom 96-well-plate) was performed on transfectants
grown in G-418 in order to isolate cell clones with
different levels of HAAH activity as measured by Western
blot analysis and enzymatic assay of hydroxylase activity.
Cloned cell lines (1.0 x 10^4 cells) were suspended in

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complete medium containing 0.4 % low-melting agarose
(SeaPlaque GTG Agarose; FMC Bioproducts, Rockland, Maine)
and laid over a bottom agar mixture consisting of complete
medium with 0.53 % low-melting agarose. Each clone was
assayed in triplicate. The clones were seeded under these
conditions and 10 days later the size (positive growth > 0.1
mm in diameter) and number of foci were determined.

Tumorigenicity in nude mice

The same clones as assessed in the anchorage
independent growth assay were injected into nude mice and
observed for tumor formation. Tumorigenicity was evaluated
using 10 animals in each of 4 groups (Charles River Labs.,
Wilmington, MA). Group 1 received 1×10^7 cells stably
transfected with mock DNA, Group 2-4 received 1×10^7 cells
of clones stable transfected with pNH376 and expressing
various levels of murine HAAH protein. Nude mice were kept
under pathogen-free conditions in a standard animal
facility. Thirty days after tumor cell inoculation, the
animals were sacrificed using isofluorane (Aerrane,
Anaquest, NJ) containing chambers and the tumors were
carefully removed and weight determined.

Animal model of bile duct proliferation

Following ligation of the common bile duct,
intrahepatic bile duct proliferation was evident at 48
hours. Tissue samples obtained 8 and 16 days following
common bile duct ligation revealed extensive bile duct
proliferation as shown in Table 3.

Table 3: Bile duct proliferation and HAAH expression
at different intervals after common bile duct ligation

Group	Surgical Procedure	Microscopy*	Immunohisto-chemistry
1	no surgery	normal	negative

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2	sham surgery	normal	negative
3	6 hours post ligation	normal	negative
4	12 hours post ligation	normal	negative
5	24 hours post ligation	normal	negative
6	48 hours post ligation	minimal bile duct prolif.	negative
7	4 days post ligation	moderate bile duct prolif.	negative
8	8 days post ligation	extensive bile duct prolif.	negative
9	16 days post ligation	extensive bile duct prolif.	negative

* Investigation was performed under light microscopy
10   following a hematoxylin and eosin staining.

Immunohistochemical staining failed to detect
presence of HAAH in proliferating bile ducts at any time.
Analysis of HAAH expression in bile ducts derived from sham
surgical controls was also negative, while all samples
15   exhibited positive immunoreactivity with control antibodies
to glyceraldehyde 3-phosphate dehydrogenase. Thus, bile
duct proliferation was not associated with increased HAAH
expression in this standard animal model system.

HAAH expression in PSC
20   The liver biopsy specimens from patients with PSC
exhibited bile duct proliferation accompanied by periductal
fibrosis and a mononuclear inflammatory cell infiltrate
without evidence of dysplasia. Adjacent sections
immunostained with the an HAAH-specific monoclonal antibody
25   had no detectable HAAH immunoreactivity in proliferating

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bile ducts.   In contrast, sections of cholangiocarcinoma
that were immunostained simultaneously using the same
antibody and detection reagents manifested intense levels of
HAAH immunoreactivity in nearly all tumor cells, whereas
5   adjacent sections of the cholangiocarcinomas exhibited a
negative immunostaining reaction with monoclonal antibody to
Dengue virus.   These findings indicate that HAAH expression
was associated with malignant transformation rather than
non-cancerous cellular proliferation of intrahepatic bile
10   ducts.

HAAH associated transformation of NIH-3T3 cells

The transforming capability of the murine and human
AAH genes, as well as the murine AAH mutant construct
without enzymatic activity were compared to mock DNA
15   (negative control) and v-Src transfected NIH-3T3 cells
(positive control).   The transforming capability of murine
AAH was found to be 2-3 times that of vector DNA control as
shown in Fig. 1.   The transforming capacity of the human
gene was greater than that observed with the murine AAH (32
20   ± 1.5 versus 13 ± 2.6 transformed foci, respectively).   The
murine and human AAH transfected cells formed large foci,
resembling those of v-Src transfected fibroblasts, compared
to the occasional much smaller foci observed in cells
transfected with vector DNA that displayed the contact
25   inhibition of fibroblast cell lines.   Parallel experiments
performed using the mutant pNH376-H660 construct without
enzymatic activity revealed no transforming activity.   This
finding indicates that the enzymatic activity of HAAH is
required for the transforming activity exhibited by the HAAH
30   gene.

Anchorage-independent cell growth assay

After transient transfection with the murine AAH
construct, several different transformed foci were isolated

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for dilutional cloning experiments to establish stable transfected cell clones with different levels of HAAH gene expression. Nine different cloned cell lines were selected for further study. The expression level of the HAAH protein

5 was determined by Western blot analysis. Clones 7 and 18 had a modest increase in HAAH protein expression, yet formed large colonies in soft agar (Fig. 2). Protein loading was equivalent in all lanes as shown by immunoblotting of the same membranes with an anti-Erk-1 monoclonal antibody. The

10 increased protein expression was associated with increased enzymatic activity as shown in Fig. 3. The capability of these clones to exhibit anchorage independent cell growth in soft agar is presented in Fig. 3. All 3 clones with increased HAAH gene expression demonstrated anchorage

15 independent cell growth compared to the mock DNA transfected clone.

Tumor formation in nude mice

The 3 clones with increased HAAH gene expression were evaluated for the ability to form tumors in nude mice.

20 Tumor size in the mouse given clone 18 was compared to a mock DNA transfected clone. Clones 7, 16 and 18 were highly transformed in this assay and produced large tumors with a mean weight of 2.5, 0.9 and 1.5 grams, respectively (Fig. 4). These data indicate that overexpression of HAAH

25 contributes to induction and maintenance of the malignant phenotype in vivo.

High level HAAH expression is indicative of malignancy

In order to determine if HAAH expression was

30 associated with malignancy rather than increased cell turnover, two models of bile duct proliferation were studied. In the animal model, ligation of the common bile duct induced extensive intrahepatic bile duct proliferation,

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yet there was no evidence of HAAH gene expression under these experimental conditions as shown in Table 3. Similarly, HAAH gene expression was assessed in a human disease model associated with bile duct proliferation since

5   PSC is an autoimmune liver disease associated with destruction as well as proliferation of the intra and extrahepatic bile ducts. PSC is premalignant disease, and a significant proportion of affected individuals will eventually develop cholangiocarcinoma. However, no evidence

10   for increased HAAH gene expression in the presence of extensive bile duct proliferation. /

Having established that HAAH protein levels were elevated in cholangiocarcinoma and not in normal or proliferating bile ducts, the role of HAAH in the generation

15   of a malignant phenotype was studied. The HAAH gene was transfected into NIH-3T3 cells and cellular changes, e.g., increased formation of transformed foci, colony growth in soft agar and tumor formation in nude mice associated with malignant transformation, were evaluated. The full-length

20   murine and human AAH genes were cloned into expression constructs and transiently transfected into NIH-3T3 cells. An increased number of transformed foci was detected in cells transfected both with the murine and human AAH genes as compared to mock DNA transfected controls. The increased

25   number of transformed foci, after controlling for transfection efficiency, was not as high compared to v-Src gene transfected cells used as a positive control. The enzymatic activity of the HAAH gene was required for a malignant phenotype because a mutant construct which

30   abolished the catalytic site had no transforming properties. Several stable transfectants and cloned NIH-3T3 cell lines with a modest increase in HAAH protein levels and enzymatic activity were established. Such cell lines were placed in

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soft agar to examine anchorage independent cell growth as another property of the malignant phenotype. All cell lines grew in soft agar compared to mock DNA transfected control, and there was a positive correlation between the cellular

5   level of HAAH gene expression and the number and size of colonies formed. Three of these cloned cell lines formed tumors in nude mice. All three cell lines with increased HAAH expression were oncogenic as shown by the development of large tumors as another well-known characteristic of the

10   transformed phenotype.

To determine whether cellular changes induced by overexpression of HAAH were related to the enzymatic function, a site-directed mutation was introduced into the gene that changed the ferrous iron binding site from

15   histidine to lysine at 660th position of mouse HAAH thereby abolishing hydroxylase activity of the murine HAAH. A corresponding mutation in HAAH is used as a dominant negative mutant to inhibit HAAH hydroxylase activity. The pNH376-H660 construct had no transformation activity

20   indicating cellular changes of the malignant phenotype induced by overexpression depends on the enzymatic activity of the protein.

Notch receptors and their ligands have several EGF-like domains in the N-terminal region that contain the

25   putative consensus sequence for beta-hydroxylation. Notch ligands are important elements of the Notch signal transduction pathway and interaction of Notch with its ligands occurs by means of EGF-like domains of both molecules. Point mutations affecting aspartic acid or

30   asparagine residues in EGF-like domains that are the targets for beta-hydroxylation by HAAH reduce calcium binding and protein-protein interactions involved in the activation of downstream signal transduction pathways.   Overexpression of

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HAAH and Notch protein hydroxylation by HAAH contributes to malignancy. Tumor growth is inhibited by decreasing Notch protein hydroxylation by HAAH

The data presented herein is evidence that
high-level HAAH expression is linked to malignant transformation. An increase in expression of the HAAH cDNA in NIH-3T3 cells induced a transformed phenotype manifested by increased numbers of transformed foci, anchorage-independent growth, and tumorigenesis in nude mice. In addition, intact HAAH-enzyme was found to be required for HAAH-associated transformation. Accordingly, inhibition of as little as 20% of endogenous HAAH enzymatic activity or expression confers a therapeutic benefit. For example, clinical benefit is achieved by 50%-70% inhibtion of HAAH expression or activity after administaration of an HAAH inhibitory compound compared to the level associated with untreated cancer cell or a normal noncancerous cell.

HAAH is regulated at the level of transcription. Only modest increases in HAAH expression and enzyme activity were required for cellular transformation. These results indicate that increased HAAH gene expression and enzyme activity contribute to the generation or maintenance of the transformed phenotype and that decreasing transcription of the HAAH gene or decreasing enzymatic activity of the HAAH gene product leads to a decrease in malignancy. Accordingly, HAAH transcription is inhibited by administering compounds which decrease binding of Fos and/or Jun (elements which regulate HAAH transcription) to HAAH promoter sequences.

Since HAAH is up-regulated with malignant transformation of bile duct epithelium, and HAAH immunoreactivity is detectable on tumor cell surface membranes, HAAH is also a molecule to which to target a

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cytotoxic agent, e.g., by linking the cytotoxic agent to a compound that binds to HAAH expressed on the surface of a tumor cell. Assay of HAAH protein levels in either biological fluids such as bile, or cells obtained by fine needle aspiration is a diagnostic marker of human cholangiocarcinoma.

Example 2: Expression of AAH and growth and invasiveness of malignant CNS neoplasms

AAH is abundantly expressed in carcinomas and trophoblastic cells, but not in most normal cells, including those of CNS origin. High levels of AAH expression were observed in 15 of 16 glioblastomas, 8 of 9 anaplastic oligodendrogliomas, and 12 of 12 primitive neuroectodermal tumors (PNETs). High levels of AAH immunoreactivity were primarily localized at the infiltrating edges rather than in the central portions of tumors. Double-label immunohistochemical staining demonstrated a reciprocal relationship between AAH and tenascin, a substrate for AAH enzyme activity. PNET2 neuronal cell lines treated with phorbol ester myristate or retinoic acid to stimulate neuritic extension and invasive growth exhibited high levels of AAH expression, whereas H_2O_2-induced neurite retraction resulted in down-regulation of AAH. PNET2 neuronal cells that stably over-expressed the human AAH cDNA had increased levels of PCNA and Bcl-2, and reduced levels of p21/Waf1 and p16, suggesting that AAH overexpression results in enhanced pathological cell proliferation, cell cycle progression, and resistance to apoptosis. In addition, the reduced levels of p16 observed in AAH-transfectants indicate that AAH over-expression confers enhanced invasive growth of neoplastic cells since deletion or down-regulation of the p16 gene correlates with more aggressive and invasive in vivo growth of glioblastomas. Increased AAH

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immunoreactivity was detected at the infiltrating margins of primary malignant CNS neoplasms, further indicating a role of HAAH in tumor invasiveness.

The following materials and methods were used to generate the data described below.

Analysis of AAH Immunoreactivity in Primary Human Malignant CNS Neoplasms:

AAH immunoreactivity was examined in surgical resection specimens of glioblastoma (N=16), anaplastic oligodendroglioma (N=9), and primitive neuroectodermal tumor (PNET; supratentorial neuroblastomas (N=3) and medulloblastomas (N=9). The histopathological sections were reviewed to confirm the diagnoses using standard criteria. Paraffin sections from blocks that contained representative samples of viable solid tumor, or tumor with adjacent intact tissue were studied. Sections from normal adult postmortem brains (N=4) were included as negative controls. AAH immunoreactivity was detected using qn HAAH-specific monoclonal antibody. Immunoreactivity was revealed by the avidin-biotin horseradish peroxidase complex method (Vector ABC Elite Kit; Vector Laboratories, Burlingame, CA) using 3-3' diaminobenzidine (DAB) as the chromogen (24) and hematoxylin as a counterstain.

Tenascin and laminin are likely substrates for AAH due to the presence of EGF-like repeats within the molecules. Double-immunostaining studies were performed to co-localize AAH with tenascin or laminin. The AAH immunoreactivity was detected by the ABC method with DAB as the chromogen, and tenascin or laminin immunoreactivity was detected by the avidin-biotin alkaline phosphatase complex method (Vector Laboratories, Burlingame, CA) with BCIP/NBT as the substrate. As positive and negative controls, adjacent sections were immunostained with monoclonal

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antibody to glial fibrillary acidic protein (GFAP) and
Hepatitis B surface antigen. All specimens were batch
immunostained using the same antibody dilutions and
immunodetection reagents.

5   Cell Lines and Culture Conditions
       Studies were conducted to determine whether AAH
expression was modulated with neurite (filopodia) extension
(sprouting) as occurs with invasive growth of malignant
neoplasms. Human PNET2 CNS-derived and SH-Sy5y
10   neuroblastoma cells were cultured and stimulated for 0, 1,
2, 3, 5, or 7 days with 100 nM phorbol 12-ester 13-acetate
or 10 μM retinoic acid to induce sprouting. In addition, to
examine the effects of neurite retraction on AAH expression,
subconfluent cultures were treated for 24 hours with low
15   concentrations (10-40 μM) of H_2O_2. For both studies, AAH
expression was evaluated by Western blot analysis using the
an HAAH-specific antibody.
       Generation of PNET2 AAH-transfected Clones
       The full-length human AAH cDNA (SEQ ID NO:3) was
20   ligated into the pcDNA3.1 mammalian expression vector in
which gene expression was under the control of a CMV
promoter (Invitrogen Corp., San Diego, CA). PNET2 cells
were transfected with either pHAAH or pcDNA3 (negative
control) using Cellfectin reagent (Gibco BRL, Grand Island,
25   NY). Neomycin-resistant clones were selected for study if
the constitutive levels of AAH protein expression were
increased by at least two-fold relative to control (pcDNA3)
as detected by Western blot analysis. To determine how AAH
overexpression altered the expression of genes that modulate
30   the transformed phenotype, the levels of proliferating cell
nuclear antigen (PCNA), p53, p21/Waf1, Bcl-2, and p16 were
measured in cell lysates prepared from subconfluent cultures
of AAH (N=5) and pcDNA3 (N=5) stably transfected clones.

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PCNA was used as marker of cell proliferation. p53, p21/Waf1, and Bcl-2 levels were examined to determine whether cells that over-expressed AAH were more prone to cell cycle progression and more resistant to apoptosis. The

5   levels of p16 were assessed to determine whether AAH over-expression has a role in tumor invasiveness.

Western blot analysis

Cells grown in 10 cm^2 dishes were lysed and homogenized in a standard radioimmunoprecipitation assay

10 RIPA buffer containing protease and phosphatase inhibitors. The supernatants collected after centrifuging the samples at 12,000 x g for 10 minutes to remove insoluble debris were used for Western blot analysis. Protein concentration was measured using the BCA assay (Pierce Chemical Co, Rockford,

15 IL). Samples containing 60 μg of protein were electrophoresed in sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and subjected to Western blot analysis. Replicate blots were probed with the individual antibodies. Immunoreactivity was detected with horseradish peroxidase

20 conjugated IgG (Pierce Chemical Co, Rockford, IL) and enhanced chemiluminescence reagents. To quantify the levels of protein expression, non-saturated autoradiographs were subjected to volume densitometry using NIH Image software, version 1.6. Statistical comparisons between pHAAH and

25 pcDNA3 transfected cells were made using Student T tests.

Antibodies

HAAH-specific monoclonal antibody generated against the FOCUS hepatocellular carcinoma cells were used to detect AAH immunoreactivity. Monoclonal antibodies to tenascin,

30 and glial fibrillary acidic protein, and rabbit polyclonal antibody to laminin were purchased from Sigma Co (St. Louis, MO). Rabbit polyclonal antibody to human p16 was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The

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5C3 negative control monoclonal antibody to Hepatitis B
surface antigen was generated using recombinant protein and
used as a negative control.

<u>AAH immunoreactivity in primary malignant brains</u>

5   <u>tumors</u>

AAH immunoreactivity was detected in 15 of 16
glioblastomas, 8 of 9 anaplastic oligodendrogliomas, and all
12 PNETs. AAH immunoreactivity was localized in the
cytoplasm, nucleus, and cell processes. The tissue

10   distribution of AAH immunoreactivity was notable for the
intense labeling localized at the interfaces between tumor
and intact brain, and the conspicuously lower levels of
immunoreactivity within the central portions of the tumors.
High levels of AAH immunoreactivity were also observed in

15   neoplastic cells distributed in the subpial zones,
leptomeninges, Virchow-Robin perivascular spaces, and in
individual or small clusters of neoplastic cells that
infiltrated the parenchyma. In contrast, AAH
immunoreactivity was not detectable in normal brain. The

20   distribution of AAH immunoreactivity appeared not to be
strictly correlated with DNA synthesis since the density of
nuclei in mitosis (1-5%) was similar in the central and
peripheral portions of the tumors.

<u>Relationship between AAH and tenascin</u>

25   <u>immunoreactivity in glioblastomas</u>

Tenascin is an extracellular matrix-associated
antigen expressed in malignant gliomas. Tenascin contains
EGF-like domains within the molecule, a substrate for HAAH
hydroxylation. To localize AAH in relation to tenascin

30   immunoreactivity in malignant brain tumors, double-label
immunohistochemical staining was performed in which AAH was
detected using a brown chromogen (DAB), and tenascin, a blue
chromogen (BCIP/NBT). Adjacent sections were similarly

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double-labeled to co-localize AAH with laminin, another EGF domain containing extracellular matrix molecule expressed in the CNS. Intense levels of tenascin immunoreactivity were observed in perivascular connective tissue and in
5 association with glomeruloid proliferation of endothelial cells. The double-labeling studies demonstrated a reciprocal relationship between AAH and tenascin immunoreactivity such that high levels of AAH were associated with low or undetectable tenascin, and low levels
10 of AAH were associated with abundant tenascin immunoreactivity. Although laminins are also likely substrates for AAH enzyme activity due to the EGF repeats within the molecules, double labeling studies revealed only low levels of laminin immunoreactivity throughout the tumors
15 and at interfaces between tumor and intact tissue.

Analysis of AAH expression in neuronal cell lines treated with PMA or RA

Neuritic sprouting/filopodia extension marks invasive growth of neoplastic neuronal cells. PMA activates
20 protein kinase C signal transduction pathways that are involved in neuritic sprouting. Retinoic acid binds to its own receptor and the ligand-receptor complex translocates to the nucleus where it binds to specific consensus sequences present in the promoter/enhancer regions of target genes
25 involved in neuritic growth. Both PNET2 and SH-Sy5y cells can be induced to sprout by treatment with PMA (60-120 nM) or retinoic acid (5-10 μM). Figs. 5A-D depict data from representative Western blot autoradiographs; the bar graphs correspond to the mean $\pm$ S.D. of results obtained from three
30 experiments. Western blot analysis with the FB50 antibody detected doublet bands corresponding to protein with an molecular mass of approximately 85 kDa. Untreated PNET2 cells had relatively low levels of AAH immunoreactivity

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(Fig. 5A), whereas untreated SH-Sy5y cells had readily
detected AAH expression (Fig. 5B). Untreated PNET2 cells
exhibited polygonal morphology with coarse, short radial
cell processes, whereas SH-Sy5y cells were slightly

5   elongated and spontaneously extend fine tapered processes.
Both cell lines manifested time-dependent increases in the
levels of AAH immunoreactivity following either RA (Figs. 5A
and 5B) or PMA (Fig. 5C) stimulation and neurite extension.
In PNET2 cells, the levels of AAH protein increased by at

10 least two-fold 24 hours after exposure to RA or PMA, and
high levels of AAH were sustained throughout the 7 days of
study. In SH-Sy5y cells, the RA- or PMA-stimulated
increases in AAH expression occurred more gradually and were
highest after 7 days of treatment (Fig. 5B).

15   To examine the effect of AAH expression on neurite
retraction, PNET2 and SH-Sy5y cells were treated with low
concentrations (8-40 μM) of H_2O_2. After 24 hours exposure
to up to 40 μM H_2O_2, although most cells remained viable
(Trypan blue dye exclusion), they exhibited neurite

20 retraction and rounding. Western blot analysis using the
FB50 antibody demonstrated H2O2 dose-dependent reductions in
the levels of AAH protein (Fig. 5D).

            Effects of AAH over-expression in PNET2 cells
            To directly assess the role of AAH overexpression in

25 relation to the malignant phenotype, PNET2 cells were stably
transfected with the human full-length cDNA with gene
expression under control of a CMV promoter (pHAAH).
Neomycin-resistant clones that had at least two-fold higher
levels of AAH immunoreactivity relative to

30 neomycin-resistant pcDNA3 (mock) clones were studied. Since
aggressive behavior of malignant neoplasms is associated
with increased DNA synthesis, cell cycle progression,
resistance to apoptosis, and invasive growth, the changes in

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phenotype associated with constitutive over-expression of
AAH were characterized in relation to PCNA, p21/Waf1, p53,
Bcl-2, and p16. PCNA was used as an index of DNA synthesis
and cell proliferation. p21/Waf1 is a cell cycle inhibitor.
5 Expression of the p53 tumor-suppressor gene increases prior
to apoptosis, whereas bcl-2 inhibits apoptosis and enhances
survival of neuronal cells. p16 is an oncosuppressor gene
that is often either down-regulated or mutated in
infiltrating malignant neoplasms.

10   Five pHAAH and 5 pcDNA3 clones were studied.
Increased levels of AAH expression in the pHAAH transfected
clones was confirmed by Western (Fig. 6) and Northern blot
analyses. Western blot analysis using cell lysates from
cultures that were 70 to 80 percent confluent demonstrated
15 that constitutively increased levels of AAH expression
(approximately 85 kDa; $P<0.05$) in pHAAH-transfected cells
were associated with significantly increased levels of PCNA
(approximately 35 kDa; $P<0.01$) and Bcl-2 (approximately
25 kDa; $P<0.05$), and reduced levels of p21/Waf1
20 (approximately 21 kDa; $P<0.001$) and p16 (approximately
16 kDa; $P<0.001$) (Fig. 6). However, the pHAAH stable
transfectants also exhibited higher levels of wild-type p53
(approximately 53-55 kDa). Although AAH expression (85 kDa
protein) in the stable transfectants was increased by only
25 75 to 100 percent, the levels of p16 and p21/Waf1 were
sharply reduced, and PCNA increased by nearly two-fold
(Fig. 6).

      <u>Increased AAH expression is indicative of growth and
invasiveness of malignant CNS neoplasms</u>
30   The data described herein demonstrates that AAH
overexpression is a diagnostic tool by which to identify
primary malignant CNS neoplasms of both neuronal and glial
cell origin. Immunohistochemical staining studies

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demonstrated that AAH overexpression was detectable mainly at the interfaces between solid tumor and normal tissue, and in infiltrating neoplastic cells distributed in the subpial zones, leptomeninges, perivascular spaces, and parenchyma. *In vitro* experiments demonstrated that AAH gene expression was modulated with neurite (filopodium) extension and invasiveness and down-regulated with neurite retraction. In addition, PNET2 cells stably transfected with the AAH cDNA exhibited increased PCNA and bcl-2, and reduced Waf1/p21 and p16 expression. Therefore, AAH overexpression contributes to the transformed phenotype of CNS cells by modulating the expression of other genes that promote cellular proliferation and cell cycle progression, inhibit apoptosis, or enhance tumor cell invasiveness.

The data demonstrated readily detectable AAH mRNA transcripts (4.3 kB and 2.6 kB) and proteins (85 kDa and 50-56 kDa) in PNET2 and SH-Sy5y cells, but not in normal brain. Correspondingly, high levels of AAH immunoreactivity were observed in 35 of the 37 in malignant primary CNS-derived neoplasms studied, whereas the 4 normal control brains had no detectable AAH immunoreactivity. The presence of high-level AAH immunoreactivity at the infiltrating margins and generally not in the central portions of the tumors indicates that AAH overexpression is involved in the invasive growth of CNS neoplasms. Administration of compounds which decrease AAH expression or enzymatic activity inhibits proliferation of CNS tumors which overexpress AAH, as well as metastases of CNS tumors to other tissue types.

The AAH enzyme hydroxylates EGF domains of a number of proteins. Tenascin, an extracellular matrix molecule that is abundantly expressed in malignant gliomas, contains EGF-like domains. Since tenascin promotes tumor cell

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invasion, its abundant expression in glioblastomas represents an autocrine mechanism of enhanced tumor cell growth vis-α-vis the frequent overexpression of EGF or EGF-like receptors in malignant glial cell neoplasms.

5 Analysis of the functional domains of tenascins indicated that the mitogenic effects of this family of molecules are largely mediated by the fibronectin domains, and that the EGF-like domains inhibit growth, cell process elongation, and matrix invasion. Therefore, hydroxylation of the

10 EGF-like domains by AAH represents an important regulatory factor in tumor cell invasiveness.

Double-label immunohistochemical staining studies demonstrated a reciprocal relationship between AAH and tenascin immunoreactivity such that high levels AAH

15 immunoreactivity present at the margins of tumors were associated with low levels of tenascin, and low levels of AAH were often associated with high levels of tenascin. These observations indicated that AAH hydroxylation of EGF-like domains of tenascin alters the immunoreactivity of

20 tenascin protein, and in so doing, facilitates the invasive growth of malignant CNS neoplasms into adjacent normal tissue and perivascular spaces.

AAH immunoreactivity was examined in PNET2 and SH-Sy5y neuronal cells induced to undergo neurite extension

25 with PMA or retinoic acid, or neurite retraction by exposure to low doses of H_2O_2. AAH expression was sharply increased by PMA- or retinoic acid-induced neurite (filopodium) extension, and inhibited by H2O2-induced neurite retraction and cell rounding. Neurite or filopodium extension and

30 attachment to extracellular matrix are required for tumor cell invasion in the CNS. The EGF-like domains of tenascin inhibit neuritic and glial cell growth into the matrix during development.

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To directly examine the role of AAH overexpression in relation to the transformed phenotype, genes modulated with DNA synthesis, cell cycle progression, apoptosis, and tumor invasiveness were examined in neuronal cell clones that stably over-expressed the human AAH cDNA. The findings of increased PCNA and reduced Waf1/p21 immunoreactivity indicated that AAH overexpression enhances cellular proliferation and cell cycle progression. In addition, the finding of increased Bcl-2 expression indicated that AAH overexpression contributes to the transformed phenotype by increasing cellular resistance to apoptosis. The apparently contradictory finding of higher levels of p53 in the cells that overexpressed AAH is explained by the observation that high levels of wildtype p53 in immature neuronal cells were associated with neuritic growth (invasiveness) rather than apoptosis. Levels of p16 were reduced (compared to normal cells) or virtually undetectable in cells that constitutively overexpressed AAH; a deletion mutation of the p16 gene has been correlated with invasive growth and more rapid progression of malignant neoplasms, including those of CNS origin. These data indicate that p16 expression is modulated by AAH.

Example 3: Increased HAAH production and IRS-mediated signal transduction

IRS-1 mediated signal transduction pathway is activated in 95% of human HCC tumors compared to the adjacent uninvolved liver tissue. HAAH is a downstream effector gene involved in this signal transduction pathway. HAAH gene upregulation is closely associated with overexpression of IRS-1 in HCC tumors as revealed by immunohistochemical staining and Western blot analysis. A high level of HAAH protein is expressed in HCC and cholangiocarcinoma compared to normal hepatocytes and bile

ducts. Both of these tumors also exhibit high level expression of IRS-1 by immunohistochemical staining. FOCUS HCC cell clones stably transfected with a C-terminal truncated dominant negative mutant of IRS-1, which blocks insulin and IGF-1 stimulated signal transduction, was associated with a striking reduction in HAAH gene expression in liver. In contrast, transgenic mice overexpressing IRS-1 demonstrate an increase in HAAH gene expression by Western blot analysis. Insulin stimulation of FOCUS HCC cells (20 and 40 U) in serum free medium and after 16 hr of serum starvation demonstrated upregulation of HAAH gene expression. These data indicate that HAAH gene expression is a downstream effector of the IRS-1 signal transduction pathway.

Example 4: Effects of HAAH expression levels on the characteristics of the malignant phenotype

Overexpression of IRS-1 in NIH 3T3 cells induces transformation. The full-length murine HAAH construct was cloned into the pcDNA3 eukaryotic expression vector. A second murine construct encoded HAAH with abolished catalytic activity due to a site directed mutation. The full-length human HAAH cDNA was cloned into the pcDNA3 expression vector as well as a plasmid that encodes v-src which was used as a positive control for transformation activity. Standard methods were used for transfection of NIH 3T3 cells, control for transfection efficiency, assays of HAAH enzymatic activity, transformation by analysis of foci formation, anchorage-independent cell growth assays and analysis of tumorigenicity in nude mice. The data indicatet hat HAAH overexpression is associated with generation of a malignant phenotype.

Table 4: Overexpression of enzymatically active HAAH indicates malignancy

cDNA	# of foci ± S.D.[b]	NIH 3T3 clone	# of colonies[e]
pcDNA3 (mock)	6.0 ± 3.3	pcDNA (mock)	0.4 ± 0.5
murine HAAH	14.0 ± 2.9	clone 18[d]	6.2 ± 2.9
mutant murine HAAH[a]	1.6 ± 1.0	clone 16[e]	4.7 ± 6.5
human HAAH	32.0 ± 5.4		
v-scr	98.0 ± 7.1		

a. enzymatically inactive HAAH
b. P<0.01 compared to mock and mutant murine HAAH
c. P<0.001 compared to mock
d. Clone 18 is a stable cloned NIH 3T3 cell line that overexpression human HAAH by approximately two fold.
e. Clone 16 is a stable cloned NIH 3T3 cell line that overexpresses human HAAH by about 50%.

These data indicate that overexpression of HAAH is associated with formation of transformed foci. Enzymatic activity is required for cellular transformation to occur. Cloned NIH 3T3 cell lines with increased human HAAH gene expression grew as solid tumors in nude mice. HAAH is a downstream effector gene of the IRS-1 signal transduction pathway.

Example 5:   Inhibition of HAAH gene expression

The FOCUS HCC cell line from which the human HAAH gene was initially cloned has a level of HAAH expression that is approximately 3-4 fold higher than that found in normal liver. To make an HAAH antisense construct, the full length human HAAH cDNA was inserted in the opposite orientation into a retroviral vector containing a G418 resistant gene, and antisense RNA was produced in the cells. Shorter HAAH antisense nucleic acids, e.g., those

corresponding to exon 1 of the HAAH gene are also used to inhibit HAAH expression.

FOCUS cells were infected with this vector and the level of HAAH was determined by Western blot analysis. A reduction in HAAH gene expression was observed. Growth rate and morphologic appearance of cells infected with a retrovirus containing a nonrelevant Green Fluorescent Protein (GFP) also inserted in the opposite orientation as a control (Fig. 8). Cells (harboring the HAAH antisense construct) exhibited a substantial change in morphology characterized by an increase in the cytoplasm to nuclear ratio as well as assuming cell shape changes that were reminiscent of normal adult hepatocytes in culture. Cells with reduced HAAH levels grew at a substantially slower rate than retroviral infected cells expressing antisense (GFP) (control) as shown in Fig. 8. A reduction in HAAH gene expression was associated with a more differentiated noncancerous "hepatocyte like" phenotype. Expression of HAAH antisense sequences are used to inhibit tumor growth rate. Reduction of HAAH cellular levels results in a phenotype characterized by reduced formation of transformed foci, low level or absent anchorage independent growth in soft agar, morphologic features of differentiated hepatocytes as determined by light and phase contrast microscopy, and no tumor formation (as tested by inoculating the cells into nude mice).
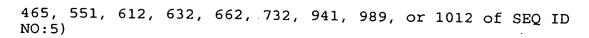
Example 6: Human IRS-1 mutants

Insulin/IGF-1 stimulated expression of HAAH in HCC cell lines. Dominant-negative IRS-1 cDNAs mutated in the plextrin and phosphotryosine (PTB) domains, and Grb2, Syp and PI3K binding motifs located in the C-terminus of the molecule were constructed. Human IRS-1 mutant constructs were generated to evaluate how HAAH gene expression is

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upregulated by activation of the IRS-1 growth factor signal
transduction cascade. Specific mutations in the C terminus
of the hIRS-1 molecule abolished the various domains which
bind to SH2-effector proteins such as Grb2, Syp and PI3K.
The human IRS-1 protein contains the same Grb2 and Syp
binding motifs of 897YVNI (underlined in Table 5, below and
1180YIDL (underlined in Table 5, below), respectively, as
the rat IRS-1 protein. Mutants of hIRS-1 were constructed
by substitution of a TAT codon (tyrosine) with a TTT codon
(phenylalanine), in these motifs by use of
oligonucleotide-directed mutagenesis suing the following
primers: (5'-GGGGGAATTTGTCAATA-3' (SEQ ID NO:8) and
5'-GAATTTGTTAATATTG-3' (SEQ ID NO:9), respectively). The
cDNAs of hIRS-1 (wild-type) and mutants (tyrosine
897-to-phenylalanine and tyrosine 1180-to-phenylalanine)
were subcloned into the pBK-CMV expression vector and
designated as hIRS-1-wt, 897F, Δ-Grb2), 1180F, and ΔSyp.

Table 5:   Human IRS-1 amino acid sequence

```
MASPPESDGF SDVRKVGYLR KPKSMHKRFF VLRAASEAGG PARLEYYENE KKWRHKSSAP 61
KRSIPLESCF NINKRADSKN KHLVALYTRD EHFAIAADSE AEQDSWYQAL LQLHNRAKGH 121
HDGAAALGAG GGGGSCSGSS GLGEAGEDLS YGDVPPGPAF KEVWQVILKP KGLGQTKNLI 181
GIYRLCLTSK TISFVKLNSE AAAVVLQLMN IRRCGHSENF FFIEVGRSAV TGPGEFWMQV 241
DDSVVAQNMH ETILEAMRAM SDEFRPRSKS QSSSNCSNPI SVPLRRHHLN NPPPSQVGLT 301
RRSRTESITA TSPASMVGGK PGSFRVRASS DGEGTMSRPA SVDGSPVSPS TNRTHAHRHR 361
GSARLHPPLN HSRSIPMPAS RCSPSATSPV SLSSSSTSGH GSTSDCLFPR RSSASVSGSP 421
SDGGFISSDE YGSSPCDFRS SFRSVTPDSL GHTPPARGEE ELSNYICMGG KGPSTLTAPN 481
GHYILSRGGN GHRCTPGTGL GTSPALAGDE AASAADLDNR FRKRTHSAGT SPTITHQKTP 541
SQSSVASIEE YTEMMPAYPP GGGSGGRLPG HRHSAFVPTR SYPEEGLEMH PLERRGGHHR 601
PDSSTLHTDD GYMPMSPGVA PVPSGRKGSG DYMPMSPKSV SAPQQIINPI RRHPQRVDPN 661
GYMMMSPSGG CSPDIGGGPS SSSSSSNAVP SGTSYGKLWT NGVGGHHSHV LPHPKPPVES 721
SGGKLLPCTG DYMNMSPVGD SNTSSPSDCY YGPEDPQHKP VLSYYSLPRS FKHTQRPGEP 781
EEGARHQHLR LSTSSGRLLY AATADDSSSS TSSDSLGGGY CGARLEPSLP HPHHQVLQPH 841
LPRKVDTAAQ TNSRLARPTR LSLGDPKAST LPRAREQQQQ QQPLLHPPEP KSPGEYVNIE 901
FGSDQSGYLS GPVAFHSSPS VRCPSQLQPA PREEETGTEE YMKMDLGPGR RAAWQESTGV 961
EMGRLGPAPP GAASICRPTR AVPSSRGDYM TMQMSCPRQS YVDTSPAAPV SYADMRTGIA 1021
AEEVSLPRAT MAAASSSSAA SASPTGPQGA AELAAHSSLL GGPQGPGGMS AFTRVNLSPN 1081
RNQSAKVIRA DPQGCRRRHS SETFSSTPSA TRVGNTVPFG AGAAVGGGGG SSSSSEDVKR 1141
HSSASFENVW LRPGELGGAP KEPAKLCGAA GGLENGLNYI DLDLVKDFKQ CPQECTPEPQ 1201
PPPPPPPHQP LGSGESSSTR RSSEDLSAYA SISFQKQPED RQ (SEQ ID NO:5; GENBANK
```
Accession No. JS0670; pleckstrin domain spans residues
11-113, inclusive; Phosphate-binding residues include 46,

465, 551, 612, 632, 662, 732, 941, 989, or 1012 of SEQ ID
NO:5)

Table 6: Human IRS-1 cDNA

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cggcggcgcg gtcggagggg gccggcgcgc agagccagac gccgccgctt gttttggttg 61
gggctctcgg caactctccg aggaggagga ggaggaggga ggaggggaga agtaactgca 121
gcggcagcgc cctccccgagg aacaggcgtc ttccccgaac ccttcccaaa cctcccccat 181
cccctctcgc ccttgtcccc tcccctcctc cccagccgcc tggagcgagg ggcagggatg 241
agtctgtccc tccggccggt ccccagctgc agtggctgcc cggtatcgtt tcgcatggaa 301
aagccacttt ctccacccgc cgagatgggc ccggatgggg ctgcagagga cgcgcccgcg 361
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tctgcgactg agctggtatt tgggcggctg gtggcggctg ggacggttgg ggggtgggag 481
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ggcggctgag aggagacttg gctctcggag gatcggggct gccctcaccc cggacgcact 781
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gatgactctg tggtggccca gaacatgcac gagaccatcc tggaggccat gcgggccatg 1801
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tctttctatt cataataaac tagatactgt tgatctttta aaaaaaaaa aaaaaaaaa 5821
aaaaaaaa (SEQ ID NO:6; GENBANK Accession No. NM 005544)
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The double mutation of tyrosine 897 and 1180 was constructed by replacement of 3'-sequences coding 897F by the same region of 1180F using restriction enzymes NheI and EcoRI, and this construct was called 897F1180F orΔGrb2 ΔSyp. The expression plasmids were under control of a CMV promoter (hIRS-1-wt, ΔGrb2, ΔSyp, ΔGrb2, ΔSyp and pBK-CMV (mock) and linearized at the 3'-end of poly A signal sequences by MluI restriction enzymes followed by purification. A similar approach was used to change the tyrosine residue to

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phenyalanine at positions 613 and 942 to create the double
PI3K mutant construct (ΔPI3K). The hIRS-1 mutants have a
FLAG epitope (DYKDDDDK (SEQ ID NO:6) + stop codon) added to
the C-terminus by PCR. This strategy allows to distinguish
the mutant protein from "wild type" hIRS-1 in stable
transfected cell lines. The mutants are used to define the
link between the IRS signal transduction pathway and
activation of HAAH as a downstream effector gene and
identify compounds to inhibit transduction along the pathway
to inhibit growth of tumors characterized by HAAH
overexpression. Antibodies or other compounds which bind to
phosphorylation sites or inhibit phosphorylation at those
sites are used to inhibit signal transduction and thus
proleferation of HAA-overexpressing tumors.

Other embodiments are within the following claims.
What is claimed is: